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Evaluation of apoptosis as a mechanism of follicular cell atresia in the ovaries of cattle (*bos indicus*) and buffalo (*bubalus bubalis*) fetuses

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Abstract

The objective of this study was to investigate the occurrence of apoptosis in the ovaries of cattle and buffalo fetuses between 4 and 8 months old by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Histological analysis of the ovarian tissue showed that apoptosis occurred at all ages evaluated, presenting a similar pattern among different fetal stages in both species. Within species, secondary follicles displayed a higher ($P < 0.05$) mean number of apoptotic follicular cells than primordial or primary follicles in cattle. In buffalo there was a similar number ($P > 0.05$) of apoptotic follicular cells among the three follicular classes compared. Comparing results between species, secondary follicles had a higher ($P < 0.05$) mean number of TUNEL positive cells in bovine fetuses; however, this difference was proportional to the larger number of follicular cells present in secondary follicles in this species. In summary, the TUNEL method was effective for the detection of apoptosis in the supporting cells of ovarian follicles from bovine and buffalo fetuses with apoptosis occurring at similar rates in both species between 4 and 8 months of gestational age. Further studies are needed to better understand the dynamics of apoptosis as a regulator of follicular atresia in fetal ovaries from these species, as well as the potential involvement of the oocyte in this process.

Keywords: apoptosis, buffalo, cattle, ovarian development, TUNEL.

Introduction

During the early stages of embryonic development in mammalian species, germ cells migrate from the yolk sac to the mesoderm-derived undifferentiated gonad. In the female fetus, these cells then differentiate into oogonia, proliferate by mitosis and enter the process of meiosis (Baker, 1963). At this point, the first and largest wave of germ cell death can be identified. The surviving oocytes can then progress to the dictyotene stage of prophase I, when meiosis is arrested until around the time of ovulation later in life (Hirshifield, 1991).

Apoptosis is an organized process of cell death involved in the homeostasis of many tissues and organs, including the ovary, where it participates in oocyte and follicular development (Hughes and Gorospe, 1991; Tilly *et al*., 1991; Vinatier and Dufour, 1996). Indeed, apoptosis plays an important role in the establishment of the adult complement of oocytes, affecting about 80% of human fetus germ cells, which fall from 7×10^6 oogonia at the beginning of pregnancy to 1×10^6 and 3×10^5 oocytes by birth and puberty, respectively (Levy, 2005). Waves of oocyte degeneration via apoptosis have been shown to occur mostly at the preleptotene and pachytene stages of the first meiotic division, as well as during the periods of primordial follicle development (Coucouvanis *et al.*, 1993; Pesce and De Felici, 1994; De Pol *et al*., 1997, 1998).

In cattle, approximately two million oogonia can be identified at the mitotic stage during fetal life. However, similarly to humans, around 90% of this population is lost with only about 200,000 primordial follicles remaining at birth (Beckers *et al*., 1996). Moreover, surges of germ cell degeneration have been described in the fetal ovaries of both cattle and buffalo during the 4th, 6th, and 10th month of pregnancy (Erickson, 1966; El-Ghannam and El-Naggar, 1974, 1975). Interestingly, the ovaries of adult buffalo contain relatively fewer primordial follicles (10,000 to 19,000) as compared to cattle (~150,000), which also translates to a lesser number of antral follicles and a higher incidence of atresia (~92-95 *vs*. 70% atresia for buffalo and cattle, respectively; Grimes *et al*., 1987; Kumar *et al*., 1997; Palta and Chauhan, 1998; Feranil *et al*., 2005). The potential physiological reason for the increased incidence of follicular atresia in the ovaries of buffalo as compared to cattle is not known. Furthermore, while this could relate to the different rates of germ cell apoptosis during fetal life between the two species, this has not been directly investigated.

Since apoptosis is characterized by an organized process that includes membrane blebbing, the activation of caspases, and DNA fragmentation, specific tests can be used to detect this process (Vinatier and Dufour, 1996). Hence, the assessment of germ cell and follicular apoptosis has been performed using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay in fetal ovaries from

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humans and sheep, as well as in adult ovaries from buffalo (Abir *et al*., 2002; Fulton *et al*., 2005; Aladaer *et al*., 2008; Sreejalekshmi *et al*., 2011). Notably, experiments in buffalo showed that in primary and secondary follicles, apoptosis started at the level of the oocyte, whereas in tertiary follicles the process began at the level of the granulosa cells (Sreejalekshmi *et al*., 2011). However, there are no reports correlating the occurrence of apoptosis with the waves of degeneration observed during follicular development in the fetal ovaries of cattle or buffalo. Therefore, the aim of this study was to evaluate the occurrence of apoptosis by means of the TUNEL assay in fetal ovaries from these species.

Materials and Methods

Collection of ovaries

Ovaries were collected from each of nine bovine and buffalo fetuses at each of 4, 5, 7, and 8 months of age. Age was stimated by measurements of the crown-rump length (Abdel-Raouf and El-Naggar, 1968; Evans and Sack, 1973).

Whole ovaries were immediately fixed in 10% paraformaldehyde for 24 h prior to processing.

Histological processing and TUNEL

Only one ovary (right or left, chosen randomly) from each animal was used for analysis in this study. After fixation, the ovaries were processed for classical histology. Sections of 7 µm were processed for TUNEL (TACS XL-BASIC ®, R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Slides were then stained with hematoxylin (Vetec, Rio de Janeiro, Brazil) and eosin (Labsynth, São Paulo, Brazil) or Methyl green (67060- Fluka, Sigma-Aldrich, São Paulo, Brazil) and examined under light microscopy (Olympus CH 30, Melville, NY, USA).

Apoptosis was subjectively scored as densely present (+++), moderately present (++), rarely present (+), or absent (–) as previously described (Feranil *et al*., 2005) by an experienced researcher blinded to slide origin, and then classified by age group within species. In addition, the number of TUNEL positive cells was counted for each follicular class (i.e. primordial, primary, and secondary). Follicles were classified according to the criteria listed in the Nomina Histologica (International Committee on Veterinary Histological Nomenclature, 1994).

Statistical analyses

Means (±SEM) for the degree of apoptosis in buffalo and cattle ovaries at different stages of development and average number of TUNEL positive cells at each stage of follicular development were compared by analysis of variance (ANOVA) using the BioEstat 5.0 software (IDSM, MCT, CNPq, Belém, Pará, Brazil; Ayres *et al*., 2007) and differences were considered significant at a $P < 0.05$. When significant differences were detected, the Tukey´s test was applied.

Results

Histological analysis of the ovarian tissue from cattle and buffalo fetuses showed that apoptosis occurred at all evaluated ages, presenting a similar pattern among different fetal ages. There was an apparent decrease in the amount of TUNEL stained tissue in 8-month-old buffalo fetuses only (Table 1). When comparing the number of follicular cells positive for the TUNEL assay among the different follicle types within species, bovine primordial and primary follicles presented a similar number ($P > 0.05$), while secondary follicles displayed a higher ($P < 0.05$) mean number of apoptotic follicular cells. In buffalo there was a similar number ($P > 0.05$) of apoptotic follicular cells among the three follicular classes compared (Table 2).

Table 1. Average degree of apoptosis as assessed by the TUNEL assay in the ovaries of cattle and buffalo at different stages of fetal life.

Fetal age	Cattle	Buffalo
4 months	++ (n = 2)	++ (n = 3)
5 months	++ (n = 3)	++ (n = 2)
7 months	++ (n = 2)	++ (n = 2)
8 months	++ (n = 2)	+ (n = 2)

n = number of fetuses. Degree of apoptosis: +++ densely present; ++ moderately present; + rarely present; – absent.

When comparing the number of apoptotic cells in the different follicle types between species, secondary follicles had a higher ($P < 0.05$) mean number of TUNEL positive cells in bovine fetuses. However, that difference was proportional to the larger number of follicular cells present in secondary follicles in this species (Table 2). Notably, apoptosis as assessed by TUNEL was only detected in follicular cells, but never within oocytes (Fig. 1 and 2).

Table 2. Mean (±SEM) number of apoptotic follicular cells in an equatorial section of fetal bovine and buffalo ovaries.

	Primordial follicle	Primary follicle	Secondary follicle
Cattle	0.5 ± 1.0^{a} n = 182 (7.5%)	0.5 ± 0.8^{a} n = 124 (3.4%)	$1.5 \pm 1.8^{b,A}$ n = 161 (2.2%)
Buffalo	0.5 ± 0.9^{a} n = 198 (7.1%)	0.4 ± 0.7^{a} n = 169 (3.3%)	$0.5 \pm 0.9^{a,B}$ n = 43 (1.7%)

n = number of follicles analyzed. ( ) Percentage in relation to the total number of follicular cells. ^{a,b}Different lowercase superscripts within a row indicate significant differences ($P < 0.01$). ^{A,B}Different uppercase superscripts within a column indicate significant differences ($P < 0.01$).

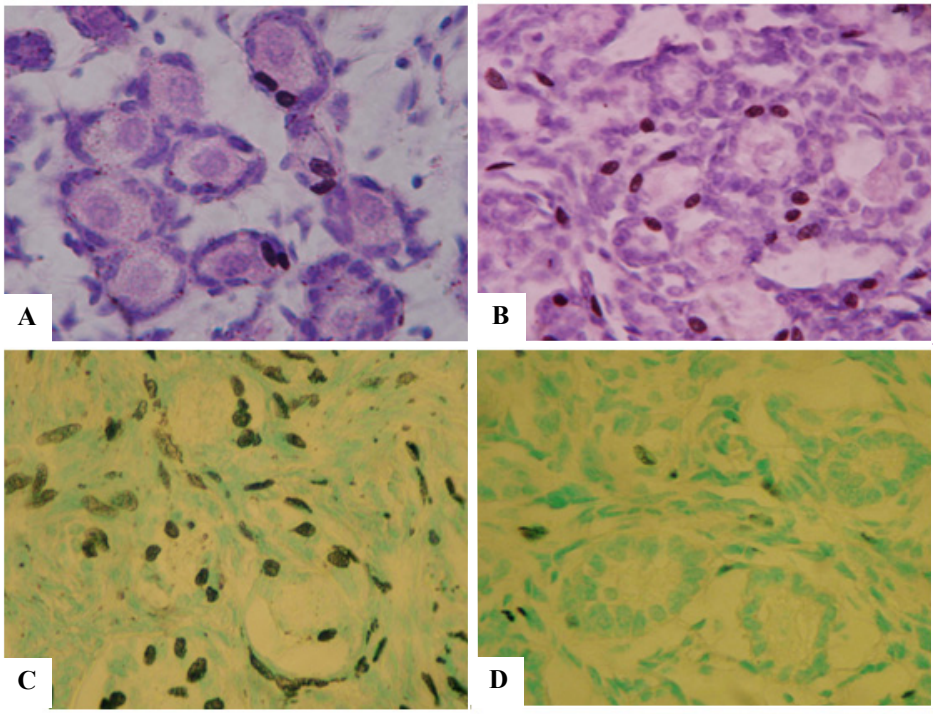

Figure 1. Ovarian sections from buffalo fetuses of different gestational ages showing cells undergoing apoptosis as assessed by TUNEL. (A) Primordial follicles, 4 months, 400X; (B) Primordial follicles, 5 months, 100X; (C) Primordial and primary follicles, 7 months, 100X; (D) Primary follicles, 8 months, 100X. (A,B) Hematoxylin and eosin; (C,D) Methyl Green.

When comparing the mean number of apoptotic follicular cells among different follicle types and fetal ages within species, the highest average number of TUNEL positive cells was observed at 8 months for primordial and secondary follicles in cattle. For primary follicles, results were similar and relatively low across all fetal ages (Table 3). In buffalo, the number of apoptotic cells was similar for both primordial and secondary follicles from 4 to 7 months of age. Conversely, primary follicles showed a higher mean number of apoptotic follicular cells at 5 and 7 months of fetal age. Interestingly, at 8 months of fetal age follicular apoptotic cells were only observed in antral follicles (data not shown).

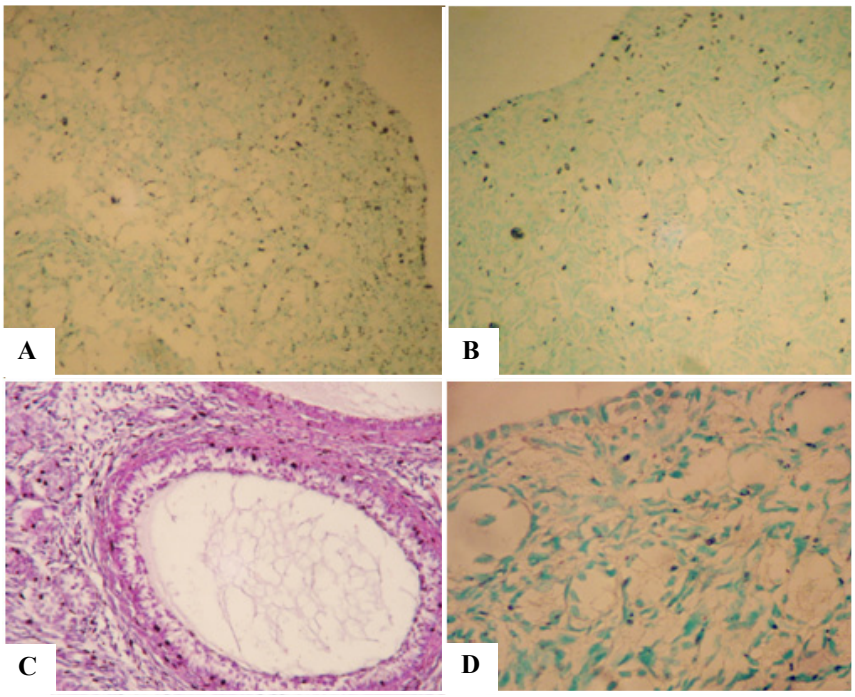

Figure 2. Ovarian sections from bovine fetuses of different gestational ages showing cells undergoing apoptosis. (A) 4 months, 40X; (B) 5 months, 40X; (C) 7 months, 40X; (D) 8 months, 100X. (A,B,D) Methyl Green; (C) Hematoxylin and eosin.

Table 3. Mean (±SEM) number of follicular cells undergoing apoptosis as assessed by TUNEL in primordial, primary and secondary follicles when evaluating the equatorial section of ovaries from cattle and buffalo fetuses of different gestational ages.

		Gestational age			
		4 months	5 months	7 months	8 months
Cattle	Primordial	0.1 ± 0.4^{a} (0-1) n = 28	0.6 ± 0.9^{a} (0-3) n = 65	0.2 ± 0.6^{a} (0-3) n = 63	1.8 ± 1.2^{b} (0-4) n = 26
	Primary	0.3 ± 0.5^{a} (0-1) n = 10	0.7 ± 0.9^{a} (0-3) n = 45	0.2 ± 0.6^{a} (0-3) n = 57	1.2 ± 1.0^{a} (0-3) n = 12
	Secondary	0.3 ± 0.5^{a} (0-1) n = 9	1.6 ± 2.5^{b} (0-10) n = 118	1.5 ± 1.3^{b} (0-6) n = 26	2.9 ± 1.8^{b} (1-6) n = 8
Buffalo	Primordial	0.3 ± 0.5^{a} (0-2) n = 47	0.5 ± 0.8^{a} (0-3) n = 52	0.3 ± 0.5^{a} (0-2) n = 64	0^{b} n = 35
	Primary	0.3 ± 0.6^{a} (0-3) n = 63	0.8 ± 0.6^{b} (0-2) n = 33	0.7 ± 1.1^{b} (0-5) n = 59	0^{c} n = 14
	Secondary	0.8 ± 1.6^{a} (0-5) n = 10	1.0 ± 1.4^{a} (0-2) n = 8	0.9 ± 1.0^{a} (0-2) n = 15	0^{b} n = 10

n = number of follicles analyzed. ( ) range of apoptotic follicular cells. [a,b]Different superscripts within a row indicate significant differences ($P \leq 0.05$).

Discussion

To our knowledge, this is the first study showing the apoptosis signals in ovarian development during fetal life in both cattle and buffalo. One hallmark of apoptosis is the presence of a typical intranuclear DNA fragmentation (Hughes and Gorospe, 1991) with the TUNEL assay providing a means to identify such changes within mammalian ovaries (De Pol *et al*., 1997; Abir *et al*., 2002). Therefore, the fact that follicular cells were marked by the TUNEL assay in this study is consistent with other reports describing waves of apoptosis in the establishment of the ovarian follicular population (Coucouvanis *et al*., 1993; De Pol *et al*., 1997; McGee *et al*., 1998; Abir *et al*., 2002; Levy, 2005; Aladaer *et al*., 2008). Moreover, apoptosis occurred in all follicle classes analyzed, suggesting a physiological rather than a random event in ovarian follicle development. Similarly, previous reports have described DNA fragmentation within granulosa and theca interna cells of both pre- and post-ovulatory follicles in adult rat, bovine, rabbit and pig ovaries (Joly *et al*., 1994; Palumbo and Yeh, 1994; Zheng *et al*., 1994; Guthrie *et al*., 1995; Nicosia *et al*., 1995).

Interestingly, TUNEL-stained follicular cells were observed in the ovaries of all fetal ages tested in this study. This disagrees with previous reports suggesting the occurrence of surges of follicular degeneration at the 4th month and then again at the 6th and 10th months of gestational age in ovaries of buffalo fetuses (El-Ghannam and El-Naggar, 1974, 1975). Indeed, in our study, apoptosis occurred most intensely in primordial and primary follicles at 5 and 8 months, and secondary follicles at 5, 7, and 8 months for cattle, as well as in primordial and primary follicles at 5 months, primary follicles at 7 months, and secondary follicles at 4, 5, and 7 months of fetal age in buffalo ovaries. In essence, these data are consistent with apoptosis occurring throughout fetal life rather than in surges, which is in agreement with findings in fetal sheep ovaries, where apoptosis was observed throughout between 37 and 99 days of gestational age, with the highest rate occurring between 58 and 73 days (Aladaer *et al*., 2008).

Notably, the fact that this study identified apoptosis within the supporting cells of all follicular stages is in agreement with what was previously reported in adult ovaries at different stages of the estrous cycle for the same two species (Feranil *et al*., 2005), suggesting that this is not a phenomenon restricted to a given follicular stage. Conversely, other studies identified TUNEL-positive cells only in atretic tertiary follicles of adult cattle and buffalo ovaries, with no apoptosis present in granulosa or theca interna cells of apparently healthy follicles (Yang and Rajamahendran, 2000; D'Haeseleer *et al*., 2005; Sreejalekshmi *et al*., 2011). The different conclusions among different studies might be the result of different sampling methods, although it seems unlikely that apoptosis would be restricted to one follicular stage. In addition, differences in the incidence and distribution of apoptosis in follicular cells might be due to the hypothesized role that programmed cell death plays in fetal versus adult tissues. That is, apoptosis during fetal development likely plays an important role in cellular "quality control" in order to eliminate cells with meiotic anomalies and cells failing to produce survival factors, hence controlling ovarian homeostasis (Monniaux, 2002). Conversely, apoptosis in adult tissues might be the result of normal follicular atresia or degeneration during the estrous cycle.

Interestingly, as in a previous reports (Ghafari *et al*., 2007), we were unable to detect apoptosis within oocytes, thus confirming that the TUNEL assay is not a good marker of apoptosis in these cells. Ovaries from human fetuses evaluated by TUNEL and immunohistochemistry against Bcl-2, a factor upstream in the apoptotic pathway, revealed no conclusive evidence that apoptosis was responsible for the extensive loss of oogonia and oocytes from mid-pregnancy until birth. Speculation that the decline in the number of germ cells is a consequence of the delicate dynamics of meiotic events, necrosis or other processes of cell death suggests the need for further studies addressing the mechanisms that regulate germ cell atresia in fetal ovaries (Abir *et al*., 2002)

In summary, the TUNEL method was effective in this study for the detection of apoptosis in the supporting cells of ovarian follicles from bovine and buffalo fetuses. The data presented are consistent with apoptosis occurring at similar rates in both species, between 4 and 8 months of age. Further studies are needed to better understand the dynamics of apoptosis as a regulator of follicular atresia in fetal ovaries from these species, as well as the potential involvement of the oocyte in this process.

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References

Abdel-Raouf M, El-Naggar MA. 1968. Biometry of the egyptian buffalo foetus. *UARJ Vet Sci*, 5:37-43.

Abir R, Orvieto R, Dicker D, Zukerman Z, Barnett M, Fisch B. 2002. Preliminary studies on apoptosis in human fetal ovaries. *Fertil Steril*, 78:259-264.

Aladaer Q, Zhang ZP, Cao GF, Zhang Y. 2008. Histological study of germ cells development and apoptosis in mongolian sheep fetal ovaries. *Anim Reprod Sci*, 103:179-186.

Ayres M, Ayres Jr M, Ayres DL, Santos AS. 2007.

BioEstat 5.0: *Aplicações Estatísticas nas Áreas das Ciências Biológicas e Médicas*. Brasília, DF: Sociedade Civil Mamirauá. 272p.

Baker TG. 1963. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc London B*, 158:417-433.

Beckers JF, Drion PV, Figueiredo JR, Goffinn L, Pirottin D, Ectors FJ. 1996. The ovarian follicle in cow. In vivo growth and in vitro culture. *Reprod Domest Anim*, 31:543-548.

Coucouvanis EC, Sherwood SW, Carswell-Crumpton C, Spack EG, Jones PP. 1993. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. *Exp Cell Res*, 209:238-247.

De Pol A, Vaccina F, Forabosco A, Cavazutti E, Marzona L. 1997. Apoptosis of germ cells during human prenatal oogenesis. *Hum Reprod*, 12:2235-2241.

De Pol A, Marzona L, Vaccina F, Negro R, Sena P, Forabosco A. 1998. Apoptosis in different stages of human oogenesis. *Anticancer Res*, 18:3457-3461.

D'Haeseleer M, Van Poucke M, Van Den Broeck W. 2005. Cell-specific localization of oestrogen receptor beta (ESR2) mRNA within various bovine ovarian cell types using in situ hybridization. *Anat Histol Embryol*, 34:265-272.

El-Ghannam F, El-Naggar MA. 1974. The prenatal development of the buffalo ovary. *J Reprod Fertil*, 41:479-483.

El-Ghannam F, El-Naggar MA. 1975. Studies of oocytogenesis of buffalo ovaries. *Zentralbl Veterinarmed A*, 22:248-255.

Erickson BH. 1966. Development and radio-response of pre-natal bovine ovary. *J Reprod Fertil*, 10:97-105.

Evans HE, Sack WO. 1973. Prenatal development of domestic and laboratory mammals: growth curves, external features and selected references. *Anat Histol Embryol*, 2:11-45.

Feranil JB, Isobe N, Nakao T. 2005. Apoptosis in the antral follicles of swamp buffalo and cattle ovary: TUNEL and Caspase-3 histochemistry. *Reprod Domest Anim*, 40:111-116.

Fulton N, Martins da Silva SJ, Bayne RA, Anderson RA. 2005. Germ cell proliferation and apoptosis in the developing human ovary. *J Clin Endocrinol Meta*b, 90:4664-4670.

Ghafari F, Gutierrez CG, Hartshorne Geraldine M. 2007. Apoptosis in mouse fetal and neonatal oocytes during meiotic prophase one. *BMC Dev Biol*, 7:87.

Grimes RW, Matton P, Ireland JJ. 1987. A comparison of histological and non-histological indices of atresia and follicular function. *Biol Reprod*, 37:1:82-88.

Guthrie HD, Cooper BS, Welch GR, Zakaria AD, Johnson LA. 1995. Atresia in follicles grown after ovulation in the pig: measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol- 17 beta. *Biol Reprod*, 52:920-927.

Hirshifield AN. 1991. Development of follicles in the mammalian ovary. *Int Rev Cytol*, 124:43-101.

Hughes FM, Gorospe WC. 1991. Biochemical identication of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology*, 129:2415-2422.

International Committee on Veterinary Histological Nomenclature. 1994. *Nomina Histologica*. 2nd ed. Zurich: ICVHN. (Together with Nomina Anatomica Veterinaria. 4th ed. and Nomina Embriologica Veterinaria).

Joly PD, Tisdall DJ, Heath DA, Lun S, McNatty KP. 1994. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3', 5'-monophosphate response to follicle-stimulating hormone. *Biol Reprod*, 51:934-944.

Kumar A, Solanki VS, Jindal SK, Tripathi VN, Jain GC. 1997. Oocyte retrieval and histological studies of follicular population in buffalo ovaries. *Anim Reprod Sci*, 47:189-195.

Lévy R. 2005. Apoptosis in oocyte. *Gynécol Obstét Fertil*, 33:645-652.

McGee EA, Hsu S-Y, Kaipia A, Hsueh AJW. 1998. Cell death and survival during ovarian follicle development. *Mol Cell Endocrinol*, 140:15-18.

Monniaux D. 2002. Oocyte apoptosis and evolution of ovarian reserve. *Gynecol Obstet Fertil*, 30:822-826.

Nicosia N, Nicosia RF, Saunders BO, Muro-Cacho C. 1995. Cell proliferation and apoptosis during development and aging of the rabbit corpus luteum. *Ann Clin Lab Sci*, 25:143-157.

Palta P, Chauhan MS. 1998. Laboratory production of buffalo (*Bubalus bubalis*) embryos. *Reprod Fertil Dev*, 10:379-391.

Palumbo A, Yeh J. 1994. In situ localization of apoptosis in the rat ovary during follicular atresia. *Biol Reprod,* 51:888-895.

Pesce M, De Felici M. 1994. Apoptosis in mouse primordial germ cells: a study by transmission and scanning electron microscope. *Anat Embryol* 189:435-440.

Sreejalekshmi P, Raghavendra BS, Subramani TS, Murthy VC, Jamuna KV, Prasad RV, Ravindra JP, Selvaraju S. 2011. Detection of follicular apoptosis in water buffalo (*Bubalus bubalis*) ovary by histology and nick end labeling technique. *Reprod Domest Anim*, 46:59-65.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ. 1991. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology*, 129:2799-2801.

Vinatier D, Dufour D. 1996. Apoptosis: a programmed cell death involved physiology in ovarian and uterine. *Eur J Obstet Gynecol Reprod Biol*, 67:85-102.

Yang M, Rajamahendran R. 2000. Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth

factor-I on spontaneous apoptosis in cultured bovine granulosa cells. *Biol Reprod*, 62:1209-1217.

Zheng J, Redmer DA, Fricke PM, Reynolds LP. 1994. Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biol Reprod*, 51:623-632.